IN SEARCH OF EXTRAMOLLUSCAN FMRFamide-RELATED PEPTIDES

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ABBREVIATIONS

Amino Acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate
E	Glu	Glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
[hP]	[hPro]	Hydroxyproline
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
pQ	pGlu	Pyroglutamate
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Other Abbreviations

ACN	Acetonitrile
BQ	Benzoquinonium chloride
CCAP	Crustacean cardioactive peptide
CCK	Cholecystokinin
CNS	Central nervous system
cpm	Counts per minute
C-terminal	Carboxy terminal
EDTA	Ethylenediaminetetraacetic acid
FAB	Fast atom bombardment
FaRP	FMRFamide-related peptide
FITC-GAR	Fluorescein isothiocyanate-conjugated goat anti-rabbit globulin
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HFBA	Heptafluorobutyric acid
HPLC	High Performance Liquid Chromatography
ir-	Immunoreactive-
N.D.	Not determined
N.R.	No response
N-terminal	Amino terminal
RIA	Radioimmunoassay
TFA	Trifluoroacetic acid
UML	Methysergide
UV	Ultraviolet

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FMRFamide-related peptides (FaRPs) have been found primarily in molluscs and arthropods. Since these phyla are thought to have evolved, with annelids, from a common ancestor, annelids should contain FaRPs. Indeed, acetone extracts of the polychaete annelid, *Nereis virens*, contained a single tetrapeptide, FMRFamide. Each worm contained 100 to 600 fmol of peptide, levels 10 to 100 times lower than in molluscs.

Immunohistochemical techniques were used to localize the peptide. FMRFamidergic cells and fibers were found in the supraesophageal (brain) and subesophageal ganglia, as well as in the intersegmental ganglia of the ventral nerve cord. Immunoreactive fibers were present in the neuropile of, and the connectives between, the supraesophageal, subesophageal, and intersegmental ganglia. In the

periphery FMRFamidergic fibers and a few cell bodies were observed in the gut. Sparse fibers were also seen in the body wall, parapodia, and cephalic palps. When the antiserum was preabsorbed with FMRFamide, no specific immunoreactivity was detected.

The esophagus of *Nereis*, isolated and suspended in a tissue bath, responded to FMRFamide with a dose-dependent relaxation; threshold was between 30 and 300 nM, and the EC_{50} was 1.55±0.60 μM. Neither methysergide nor benzoquinonium modified this response.

In conclusion, FMRFamide is a neurotransmitter in both the central and peripheral nervous systems of *Nereis virens* and may be involved in controlling digestive tract motility.

In arthropods, the FaRPs fall into three subgroups based upon sequence; two of these groups occur in insects, but only one in crustaceans. Still, FaRPs from only a single crustacean species have been characterized. Therefore, to determine the variability of this peptide family in Crustacea, FaRPs from the pericardial organs and thoracic ganglia of the blue crab *Callinectes sapidus* were isolated and sequenced. Multiple peaks of immunoreactivity were present, and one yielded the sequence, GYNRSFLRFamide. Each animal contained between 7 and 13 pmol.

The peptide caused a dose-dependent increase in heart rate; threshold was 10 to 30 nM, and the EC_{50} was 323±62 nM. A structure-activity study of the crab heart suggests that, for full potency, the peptide should be at least a heptapeptide with the sequence XXZFLRFamide, where X is any amino acid and Z is either asparagine or serine.

CHAPTER 1

INTRODUCTION

Background

Neuropeptides can be grouped in subsets based upon various criteria; e.g., structure, function, or the tissue or species in which they occur. A group of neuropeptides with similar sequences constitute a family which, in turn, can be subdivided into a nuclear- and extended sub-families. Members of the nuclear family contain a common core sequence which is required for biological activity; the remainder of the sequence differs from the others in the sub-family in only a few extra amino acid residues. In peptides of the extended sub-family, the common core sequence is much reduced, and the remainder of the sequence is much more variable.

The family of the FMRFamide-related peptides (FaRPs) is exemplary. The eponymous tetrapeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) was first isolated from the ganglia of a clam, *Macrocallista nimbosa* (Price and Greenberg, 1977), and is present in all classes of the phylum Mollusca (Price et al., 1987). The nuclear family in molluscs includes, at present, ten additional peptides in four groups (Table 1-1). One group, comprises only

Table 1-1. INVERTEBRATE FaRPs

PHYLUM/SPECIES	PEPTIDE SEQUENCE	REFERENCE
Mollusca		
All	Phe-Met-Arg-Phe-NH_2	
	Phe-Leu-Arg-Phe-NH_2	Price et al., 1987
Pulmonates	pGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	
	Ser-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	
	Gly-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	
	Asn-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	Price et al., 1987
	Ser-Glu-Pro-Tyr-Leu-Arg-Phe-NH_2	
	Asn-Asp-Pro-Tyr-Leu-Arg-Phe-NH_2	Price et al., 1990
Octopus	Ala-Phe-Leu-Arg-Phe-NH_2	
	Thr-Phe-Leu-Arg-Phe-NH_2	Martin & Voigt, 1987
Arthropoda		
Homarus americanus	Ser-Asp-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	
	Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	Trimmer et al., 1987
Leucophaea maderae	pGlu-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2	Holman et al., 1986
Schistocerca gregaria	Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2	Robb et al., 1989
Drosophila melanogaster	Asp-Pro-Lys-Gln-Asp-Phe-Met-Arg-Phe-NH_2	Nambu et al., 1988
Nematoda		
Ascaris suum	Lys-Asn-Glu-Phe-Ile-Arg-Phe-NH_2	Cowden & Stretton, 1989
Panagrellus redivivus	Ser-Ala-Asp-Pro-Asn-Phe-Leu-Arg-Phe-NH_2	Price, personal communication
	Ser-Asp-Pro-Asn-Phe-Leu-Arg-Phe-NH_2	

FMRFamide and another tetrapeptide -- FLRFamide -- in which the methionine has been replaced by a leucine residue. This group is ubiquitous. Four heptapeptides of the form XDPFLRFamide (where X is Gly, Ser, Asn, or pGlu) are present in only pulmonate gastropods (e.g., land snails and slugs); all are N-terminally extended analogs of FLRFamide, and they only differ in their N-terminal residue (Price et al., 1987). The beginning of an analogous set of pulmonate peptides XXPYLRFamide (where XX is Ser-Glu or Asn-Asp) has been discovered in *Helix* (Price et al., 1990). Finally, the cephalopod mollusc *Octopus* seems to contain AFLRFamide and TFLRFamide (Voigt and Martin, 1986; Martin and Voigt, 1987).

Members of this nuclear sub-family are also present in other invertebrate phyla (Table 1-1). In the Arthropoda, peptides containing the core sequence FXRFamide (where X is Leu, Met, or Ile) have been sequenced: TNRNFLRFamide and SDRNFLRFamide from the lobster *Homarus americanus* (Trimmer et al., 1987); pQDVDHVFLRFamide from the cockroach *Leucophaea maderae* (Holman et al., 1986); PDVDHVFLRFamide from the locust *Schistocerca gregaria* (Robb et al., 1989); and DPKQDFMRFamide from the fruit fly *Drosophila melanogaster* (Nambu et al., 1988). Finally, among the nematodes, *Ascaris suum* contains the neuropeptide KNEFIRFamide (Cowden et al., 1989) and *Panagrellus redivivus*,

SADPNFLRFamide and SDPNFLRFamide (D. Price, personal communication). Although these peptides of the nuclear sub-family are all quite similar, no particular sequence has ever appeared without modification in two different phyla.

Molluscs and arthropods, along with annelids are believed to have evolved from a common flatworm-like ancestor (Barnes, 1974; Vagvolgyi, 1967; Stasek, 1972). This notion is based mainly on similarities of embryology and anatomy. Embryologically, members of all three phyla are characterized by spiral, determinate cleavage. The first opening to the digestive tract to develop during gastrulation is the mouth, or protostome. Annelids and molluscs also share a very similar larval form, the trochophore.

Anatomically, annelids and arthropods display segmentation, as do very primitive molluscs, like the monoplacophoran *Neopilina galatheae* (Lemche and Wingstrand, 1959). The metamerism in *Neopilina* involves, not only the musculature, but also the nervous system (Lemche and Wingstrand, 1959). However, the ontogeny of segmentation is different in each phylum and appears to have been a recent evolutionary acquisition.

Annelid and arthropod nervous systems are very similar. They consist anteriorly, of a dorsally located brain (supraesophageal ganglion) united by

circumesophageal connectives to a subesophageal ganglion; the ganglia and connectives constitute the circumesophageal nerve ring. The ring is connected, in turn, to a ventral nerve cord with ganglionic swellings in each segment (Manton, 1970). Primitive molluscs, e.g., monoplacophorans and polyplacophorans (chitons), also have a circumesophageal nerve ring, but their nerve cords lack arthropod-like segmental ganglia.

According to recent comparisons of 18S ribosomal RNA, annelidan RNA sequences are more similar to the molluscan ones than to those of arthropods (Field et al., 1988). The resulting evolutionary tree suggests that the arthropods diverged from annelids and molluscs before the latter phyla diverged from each other.

The basis for the suggestion that these three phyla evolved from a common flatworm-like ancestor comes from: (1) the similarities of flatworm embryology to that of the annelids, molluscs, and to a lesser extent arthropods, and (2) the simpler, more primitive organization of flatworm anatomy.

Genes encoding FaRP precursors have been isolated from and sequenced in three gastropod and two arthropod species. In the gastropods, three different genes have been identified: in *Aplysia*, a gene encoding 28 copies of FMRFamide and one of FLRFamide (Taussig and Scheller, 1986); in *Lymnaea*, a tetrapeptide gene encoding nine

copies of FMRFamide and two of FLRFamide (Linacre et al., 1989); and in *Helix*, a single gene encoding ten copies of FMRFamide and one copy of FLRFamide (Lutz et al., 1990). Also in *Helix*, a second separate gene has been found to encode multiple copies of some of the pulmonate heptapeptides (Lutz, personal communication). Thus in gastropod molluscs, two separate genes seem to encode the tetrapeptides and heptapeptides.

The FaRP genes of the congeneric insects *Drosophila melanogaster* and *Drosophila virilis* contain copies of several N-terminally extended analogs of FMRFamide (Schneider and Taghert, 1988; Taghert and Schneider, 1990). They have two characteristics in common with all of the molluscan genes studied so far: first, they encode for at least two different peptides; and second, they include multiple copies of <u>at least</u> one of the peptide sequences. There are still other peptides the genes of which have yet to be isolated. The discovery of such genes will probably point to further sequences which have not yet yielded to classical peptide isolation, purification, and sequencing strategies.

Immunocytochemistry and bioassay suggest several biological functions for the FaRPs in the nuclear sub-family. Initially characterized as cardioregulators, these peptides modulate both cardiac and non-cardiac muscles of molluscs (Painter and Greenberg, 1982; Lehman

and Greenberg, 1987; Krajniak and Bourne, 1987; Payza, 1987), arthropods (Trimmer et al., 1987; Cuthbert and Evans, 1989, Mercier et al., 1989), and annelids (Kuhlman et al., 1985a; 1985b; Li and Calabrese, 1987; Diaz-Miranda et al., 1989; Fujii et al., 1989; Calabrese and Norris, 1989), as well as the musculature of nematodes (Smart et al., 1990). In addition, they appear to control the digestive and reproductive states of pulmonate molluscs (reviewed in Krajniak et al., 1989). The FaRPs also affect nerve cells in molluscs (reviewed in Kobayashi and Muneoka, 1989), arthropods (Weimann and Marder, 1989), annelids (Calabrese and Norris, 1989), and nematodes (Cowden et al., 1989).

The extended sub-family of FaRPs contains peptides that end in Arg-Phe-NH_2 (RFamide); this C-terminal dipeptide is the minimal portion of the core required for cross-reactivity with FMRFamide antisera. Their cross-reactivity in standard FMRFamide bioassays (e.g., clam heart) is usually very poor. To date, these peptides have been identified primarily in cnidarians and chordates: pQGRFamide and pQLLGGRFamide are present in the cnidarians (Grimmelikhuijzen and Graff, 1986; Grimmelikhuijzen et al., 1988); the vertebrate peptides include LPLRFamide in the chicken brain (Dockray et al., 1983), gamma-1-MSH (YVMGHFRWDRFamide) in the ox pituitary (O'Donahue et al., 1984; Triepel and Grimmelikhuijzen,

1984), and the morphine modulating peptides, FLFQPQRFamide and AGELSSPFWSLAAPQRFamide in the cow brain (Yang et al., 1985).

Three members of the extended family occur in protostomous invertebrates: the L5 peptide of the mollusc *Aplysia californica* that ends in QGRFamide (Shyamala et al., 1986); and two peptides found in mosquito heads, pQRP[hP]SLKTRFamide and TRFamide (Matsumoto et al., 1989).

A variety of other peptides which are shirttail relations of the FaRP family (Price and Greenberg, 1989). They include predicted peptide sequences from genes like pQDPFLRIamide from the *Helix* heptapeptide gene, and SRPQDPVRSamide from the gene of *Drosophila*. Other members of this group come from a family of peptides in starfish and include SALMFamide, GFNSALMFamide, and SGPYSFNSGLTFamide. These peptides were isolated using an antiserum to the heptapeptide pQDPFLRFamide (Elphick et al., 1989). Their cross-reactivity with the FMRFamide receptor is likely to be extremely poor.

In conclusion, the nuclear sub-family FaRPs are restricted to protostomes, whereas the extended sub-family is ubiquitous in metazoans. Since most of the parasites and pests that plague man and commercially important vertebrates are protostomes, studies of different FaRPs and their receptors could yield new forms of antihelimenthics, insecticides, and other regulatory agents.

Objectives

The first objective of this study was to extend the boundaries of the protostomous nuclear sub-family of FaRPs. Since no peptides of annelid worms have been sequenced, extracts of the polychaete *Nereis virens* were examined for novel immunoreactive peptides.

The second objective was to determine the physiological function of the nereid FaRP. To this end, the distribution of the peptide was mapped immunohistochemically in both nervous and non-nervous tissues. Also, bioassays were performed on immunoreactive tissues, particularly the esophagus.

The third objective was to explore the variations in sequence in the nuclear sub-family of FaRPs in the superphylum, Arthropoda. Several species of insect, but only one species of crustacean, *Homarus americanus*, have yielded peptide sequences. Therefore, FaRPs of the blue crab *Callinectes sapidus* were isolated and sequenced.

Since the crustacean FaRPs are cardioexcitatory, the final objective was to study the structure-activity relationships of these peptides on the crustacean heart.

CHAPTER 2

THE FMRFamide-RELATED PEPTIDE OF *NEREIS VIRENS*

Introduction

Most of the known invertebrate FaRPs have been discovered in molluscs and arthropods. The annelids, are a closely related phylum, and ample immunohistochemical and pharmacological data suggests that FaRPs are present in all three classes of Annelida (Kuhlman et al., 1985a,b; Li and Calabrese, 1987; Porchet and Dhainaut-Courtois, 1988; Diaz-Miranda et al., 1988; 1989; Fujii et al., 1989; Calabrese and Norris, 1989). Nevertheless, no FMRFamide-related peptides in annelids have actually been sequenced.

All annelids are segmented worms, but the three classes in the phylum -- Polychaeta, Oligochaeta, and Hirudinea -- are quite different (Clark, 1978). The most variable of these classes is the Polychaeta, which includes mostly marine worms, distributed in many families, and characterized by a mixture of primitive and advanced features (Fauchild, 1977). The Oligochaeta comprise mostly terrestrial and freshwater worms, are much less diverse in structure than the polychaetes, and can readily be systematized according to their embryology and the organization of their reproductive systems

(Brinkhurst, 1982). The evolutionary connection between the oligochaetes and polychaetes is unclear, but the leeches, constituting the class Hirudinea are very closely allied to the oligochaetes, from which they diverged relatively recently. The members of this class are the least diverse and the most specialized of the three.

The role of FMRFamide in the leech *Hirudo medicinalis* has been well studied (Kuhlman et al., 1985a,b; Li and Calabrese, 1987; and Calabrese and Norris, 1989). FMRFamidergic cell bodies, processes, and varicosities are present in the brain and all segmental ganglia. The amount of ir-FMRFamide declines in the two ganglia nearest each end of the animal (head and tail). The function of many of the neurons in each leech ganglion have been identified: the immunoreactive cells include heart excitor motor neurons, heart accessory motor neurons, swim-initiating interneurons, excitatory body wall motor neurons, and the rostral and lateral penile everter motor neurons (Kuhlman et al., 1985a,b; Li and Calabrese, 1987; and Calabrese and Norris, 1989). Furthermore, a peak of immunoreactive-FMRFamide (ir-FMRFamide) has been extracted from nervous tissue of the leech and chromatographically characterized as FMRFamide; but it has yet to be sequenced (Li and Calabrese, 1987).

Application of exogenous FMRFamide to the CNS mimics the effects of the heart motor neurons and the

swim-initiating interneurons, both of which can accelerate the central pattern generator of the heartbeat. Furthermore, the peptide also stimulates the heart in the same way that the heart accessory motor neuron does. Bath application of FaRPs to isolated body wall longitudinal muscles causes a dose-dependent increase in tone. Thus, FMRFamide is involved both centrally and peripherally in the control of the leech heart and body wall musculature.

Hirudo medicinalis is a highly evolved, specialized worm, and the distribution and actions of FMRFamide in the leech may not, therefore represent those in the remaining two classes of annelids. The polychaetes are considered more primitive, and an examination of their peptides would create a more general understanding of the sequences, distributions, and functions of FaRPs in annelids. Because FMRFamide immunoreactive cell bodies were found in the brain of the polychaete *Nereis diversicolor* (Porchet and Dhainaut-Courtois, 1988), I began to isolate the FaRPs from the related species *Nereis virens*, a polychaete that is commercially available in very large numbers.

As an approach to the physiological roles of the nereid FaRP, the distribution of the peptide was mapped immunohistochemically. Furthermore, bioassays were performed on the esophagus, a tissue containing FMRFamide-immunoreactivity.

Methods

Animals

Nereis virens was purchased from F.H. Hammond Wholesale (Wiscasset, ME). The animals were maintained in flowing natural seawater at 15° C until used.

Peptide Extraction, Purification, and Sequencing

The anterior 3 cm of the worms (total length, about 20 cm) were cut off and placed in 4 volumes of acetone; the tissue was allowed to steep overnight at -20° C. The anterior 3 cm was used because it contained the circumesophageal nerve ring and, in a preliminary experiment, very little immunoreactivity was extracted from the remaining 17 cm of the body.

The acetone was decanted and driven off by rotary evaporation. The remaining aqueous residue was clarified by centrifugation and filtration. An equal volume of water was added to this fluid and then separated by HPLC. Two extracts were made and purified: a preliminary one of 500 worms, and a definitive one of 2000 worms.

The aqueous residue from the 500 animal extract (200 ml) was loaded onto a Waters Radial Pak C-18 reverse-phase HPLC column (8 x 100 mm) at a flow rate of 10 ml/min. The material was then eluted from the column with a gradient of acetonitrile (ACN) (16 to 32% in 20 min) containing 0.1% trifluoroacetic acid (TFA). One-minute fractions were collected and the ir-FMRFamide determined

by radioimmunoassay (RIA; described below). A rabbit polyclonal antiserum (S253) made to YGGFMRFamide was used in the RIA (Price et al., 1987). The few fractions containing the most immunoreactivity were diluted with water and pumped onto a Waters Novapak C-18 reverse-phase HPLC column (3.9 x 150 mm) which was eluted with a gradient of n-butanol (2 to 8% in 20 min) containing 0.1% TFA. Fractions were collected every 30 seconds, and the pooled immunoreactive fractions were further purified on the same column, except that the ACN/TFA gradient already described was used. The two buffer systems were used thus, in alternation, until the immunoreactive peak corresponded to a single peak of UV absorbance at 210 nm. The sample was then sequenced by the Protein Chemistry Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research.

The 2000 worm extraction yielded a liter of aqueous residue which was purified, 200 ml at a time, on a Brownlee Prep 10 Aquapore Octyl C-8 reverse-phase HPLC column (10 x 100 mm). The same ACN/TFA gradient was used, but with a flow rate of 4 ml/min. The immunoreactive fractions from all five runs were pooled and loaded on the same column once again. All subsequent steps were performed on a Brownlee RP-300 Aquapore Octyl reverse-phase HPLC column (2.1 x 220 mm) with a flow rate of 0.5 ml/min.

Since alternation of the ACN/TFA and butanol/TFA systems could not separate a peptide to purity, an additional step was added. The sample was neutralized to pH 7 with phosphate buffer; it was loaded onto the RP-300 column, and the immunoreactive material was eluted from the column with a gradient from 5 mM sodium phosphate (pH 7.0) to 5 mM phosphoric acid (pH 3.0), both containing 25% ACN. Although this step was inefficient -- a large portion of the immunoreactivity being eluted with the load fraction -- it gave a high degree of purification. Nevertheless, the retained immunoreactive peak was still not pure, as evidenced by the presence of 280 nm absorbance in the immunoreactive fraction. Therefore, the fractions were oxidized with 50 µl/ml of hydrogen peroxide (30%) for 15 min at room temperature. The oxidized peptide eluted at 4.51 min, several minutes earlier than the unoxidized peptide, and well before the impurities at 6.5 min.

The immunoreactivity in the final peak was measured with both the standard S253 antiserum and another polyclonal antiserum (Q2) raised in a rabbit to pQDPFLRFamide.

The purified, oxidized peptide was divided into two equal portions; one was sequenced at the University of Florida, and the other was analyzed by fast atom bombardment mass spectrometry (T.D. Lee, Division of

Immunology, Beckman Research Institute of the City of Hope, Duarte, California), as previously described (Bulloch et al.,1988).

Radioimmunoassay

The antisera used in the RIA were raised in rabbits to either YGGFMRFamide conjugated with thyroglobulin (identified as S253; Price et al., 1987) or pQDPFLRFamide conjugated with thyroglobulin (identified as Q2). The trace was iodinated pQYPFLRFamide.

The cross-reactivity of S253 and, to a lesser extent, Q2 have been examined with a number of peptides (Greenberg et al., 1988; Elphick et al., 1989). With S253 FMRFamide and the pulmonate heptapeptides are equipotent, whereas FLRFamide is one order of magnitude less potent. Other substitutions of the amino acids in tetrapeptide structure also decrease the potency of the analog. Replacing the N-terminal phenylalanine decreases the potency by two to three orders of magnitude, while replacement of the methionine in position 2 causes only a decrease of one order of magnitude. Substitution of the C-terminal phenylalanine also drops the potency by three orders of magnitude. Peptides without the C-terminal amide have no activity. Unrelated peptides, like SCP_B which has a C-terminal sequence of FPRMamide, show no cross-reactivity. Thus, S253 binds preferentially to FMRFamide

and to N-terminally extended peptides with the C-terminal sequence FXRFamide (where X is leucine or methionine).

Q2 reacts only poorly with FMRFamide-like peptides containing methionine sulfoxide in the second position. It also cross-reacts with SALMFamide, GFNSALMFamide, and SGPYSFNSGLTFamide which are shirttail FaRPs from echinoderms. Thus, the specificity of Q2 is for peptides with the C-terminal sequence LXFamide, where X is any amino acid.

The RIA was performed as follows. An aliquot (2 µl) of each HPLC fraction was transferred to a glass test tube with an automatic diluter (Micromedic model 3000) that added 48 µl RIA buffer to give a final sample volume of 50 µl. The RIA buffer included 0.01 M sodium phosphate containing 1% bovine serum albumen, 0.9% sodium chloride, 0.01% merthiolate, and 0.025 M sodium EDTA, and was adjusted to pH 7.5. For sample dilution, a buffer without albumen was used to avoid contaminating the fraction with anything containing amino acids. The trace (10,000 cpm) in 100 µl of buffer was added to each tube along with 100 µl of diluted antiserum (1:10,000 for S253, and 1:500 for Q2). All of the tubes were left overnight in the refrigerator (4° C), and 1 ml of charcoal solution was added in the morning. The charcoal solution contained 0.25% charcoal, 0.025% dextran, and 0.01% merthiolate in 0.1 M sodium phosphate, pH 7.5; it was stirred overnight

before the first use, and was kept in the refrigerator thereafter. After 15 minutes, the charcoal was centrifuged at 2500 x g for 15 minutes, and the supernatant was then decanted and counted.

Immunohistochemistry

The desired tissues were removed and fixed in Bouin's fixative overnight in the refrigerator. Following the fixation, most of the tissues were embedded in paraffin (Humason, 1967), sectioned, rehydrated, and stained by the indirect immunofluorescence method (Beltz and Burd, 1989). Ganglia to be wholemounted were desheathed the day after fixation, washed, and stained according to the wholemounting procedures outlined in Beltz and Burd (1989), except that 1% goat serum was added to both the primary and secondary antisera to block non-specific immunoreactivity. All tissues were stained with a different polyclonal serum raised in rabbits to FMRFamide (1:1000 dilution; supplied by Dr. E. Weber, Oregon Health Science University, Portland, OR). Control tissues were stained with the same antiserum (1:1000 dilution) preabsorbed with FMRFamide (1 mg/ml). The secondary antibody in all cases was fluorescein isothiocyanate-conjugated goat anti-rabbit gamma globulin (FITC-GAR, 1:50 dilution).

The specificity of the Weber antiserum differs from those of S253 and Q2 in that it cross-reacts poorly with

even close analogs of FMRFamide or YGGFMRFamide (Price, personal communication). Peptides containing the C-terminal sequence FXRFamide, where X is leucine or proline, are at least two orders of magnitude less potent when this serum is used in the FMRFamide RIA. Nevertheless, this antiserum always shows more immunoreactivity than S253 in immunohistochemistry (personal observation), and is widely used.

Bioassay

The esophagus, unlike other parts of the digestive tract, is unattached to the body wall and was therefore chosen for the bioassay. Contractions of the isolated esophagus were recorded as follows. The portion of the esophagus distal to the pharynx anteriorly, and proximal to the intestine posteriorly, was removed; the esophageal ceca (digestive glands) were left attached. Ligatures were then tied around both ends of the tissue, and the posterior end was secured to a stationary rod, while the anterior was connected to a force-displacement transducer (Grass FT-03) coupled to a Grass Model 7 Polygraph. In some experiments, the esophagus was stimulated with concentric platinum electrodes on the stationary rod connected to a Grass stimulator (2 Hz, 1 ms, 20 V) (Figure 2-1). All drugs were added directly to the organ bath from stock solutions; the dose reported was the final molar concentration in the bath.

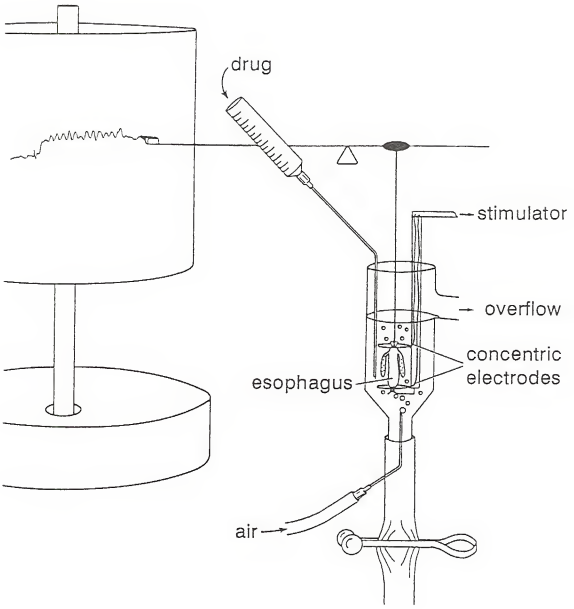
Results

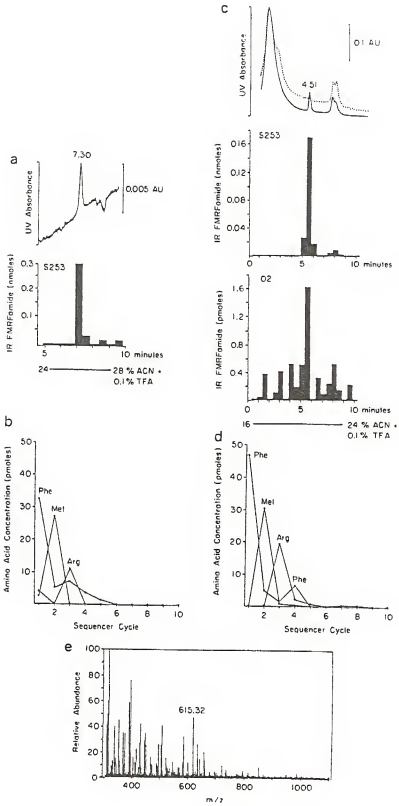
Peptide Extraction, Purification, and Sequencing

The initial fractionation of the extract yielded a single peak of immunoreactivity, irrespective of the column used. But subsequent steps often showed a minor peak of immunoreactivity corresponding to the oxidized form of the peptide. The initial peak from the first batch of 500 worms contained about 2.0 nmol of ir-FMRFamide. The purified peak of immunoreactivity (Fig. 2-2a) was equivalent to about 600 fmol/worm, but the UV absorbance was more indicative of about 300 fmol/worm, and the sequencing levels were those expected for about 150 fmol/worm.

From the second extract, containing 2000 worms, the initial amount of ir-FMRFamide from the pooled first run was 15.4 nmol. In the final step (Fig. 2-2c), I recovered about 300 fmol/worm according to the RIA data, 250 fmol/worm from the UV data, and 100 fmol/worm from sequencing. In this instance, the amount of peptide is smaller than that in the 500 worm batch, but much of the peptide was lost when the sample was separated on the pH gradient.

Figure 2-1. The isolated *Nereis* esophagus bioassay apparatus. The figure shows a schematic of the isolated esophagus suspended in a tissue bath connected at its posterior end to an hook in the electrode support and at its anterior end to a transducer. Concentric electrodes surround the tissue and were used with a Grass stimulator to stimulate a tonic contraction (2 Hz, 1 ms, 20 V). In this drawing the recording device shown is a smoked-drum kymograph, but in reality a Grass transducer and recorder were used. The same apparatus was used whether the tissue was electrically stimulated or not. All drugs were added directly to the organ bath from stock solutions; the dose reported was the final molar concentration in the bath.


drug
stimulator
overflow
concentric
electrodes
esophagus
air


a
7.30
UV Absorbance
0.005 AU
IR FMRFamide (nmoles)
S253
0.3
0.2
0.1
5
10 minutes
24
28 % ACN +
0.1 % TFA
b
Amino Acid Concentration (pmoles)
50
40
30
20
10
Phe
Met
Arg
2
4
6
8
10
Sequencer Cycle
c
UV Absorbance
0.1 AU
4.51
d
IR FMRFamide (nmoles)
S253
0.16
0.12
0.08
0.04
0
5
10 minutes
IR FMRFamide (pmoles)
02
1.6
1.2
0.8
0.4
16
24 % ACN +
0.1 % TFA
Amino Acid Concentration (pmoles)
Phe
Met
Arg
Phe
Sequencer Cycle
e
Relative Abundance
100
80
60
40
20
0
615.32
400
600
800
1000
m/z

The antisera S253 and Q2 indicated different levels of ir-FMRFamide in the major peak of the 2000 animal extraction. The level assayed with S253 was 0.16 nmol, whereas it was 1.6 pmol with the Q2 antiserum, a difference of 100-fold. The same differential occurs when the methionine in FMRFamide is oxidized to the sulfoxide. Thus, these data suggested that the peak contained an oxidized peptide with the C-terminal sequence of Met-Arg-Phe-NH_2.

The sequence of both samples can be seen in Figures 2-2b and 2-2d. The sequence from the 500 animal extract clearly contains the tripeptide, Phe-Met-Arg. Since the S253 antiserum requires an amidated C-terminal phenylalanine for binding in the RIA (Price, 1983; Greenberg et al., 1988; Price, 1987), this tripeptide could not constitute the complete sequence. Indeed, in the analysis of the oxidized peak from the 2000 worm extract, the peak had the sequence Phe-Met-Arg-Phe. The presence of the C-terminal amide was indicated by the weight of the molecular ion of the oxidized sample as determined by FAB-mass spectrometry (Fig. 2-2e). The observed molecular ion was 615.32 which is extremely close to the calculated weight 615.31 of oxidized FMRF-NH_2 (the calculated molecular ion of oxidized FMRF-OH is 616.31). Thus, the FaRP of *Nereis virens* is FMRFamide itself.

Immunohistochemistry

The central nervous system of *Nereis virens* consists of a brain (supraesophageal ganglion) connected to a subesophageal ganglion via the paired circumesophageal connectives (Figure 2-3). The subesophageal ganglion is continuous with the ventral nerve cord which joins the intersegmental ganglia. The subesophageal ganglion itself is composed of three fused intersegmental ganglia.

Immunoreactive FMRFamide was found in cell somas throughout the central nervous system. These bodies were distributed in a bilaterally symmetrical pattern in all ganglia (supraesophageal, subesophageal, and intersegmental). The cell bodies ranged from 10 to 85 μm in diameter, depending upon their locations, and each ganglion appeared to have at least 50 or more immunoreactive cells. Assuming that the anterior 3 cm of the worm contained 1000 spherical cells in the circumesophageal ring and ventral nerve cord, an average cell diameter of about 35 μm, and an intracellular concentration of about 1mM FMRFamide, the total amount of peptide in this section of the worm would have been about 180 fmol. This estimation was within the range determined by RIA of acetone extracts (i.e., 100-600 fmol/worm). Thus, the immunohistochemical data were consistent with those from peptide isolation.

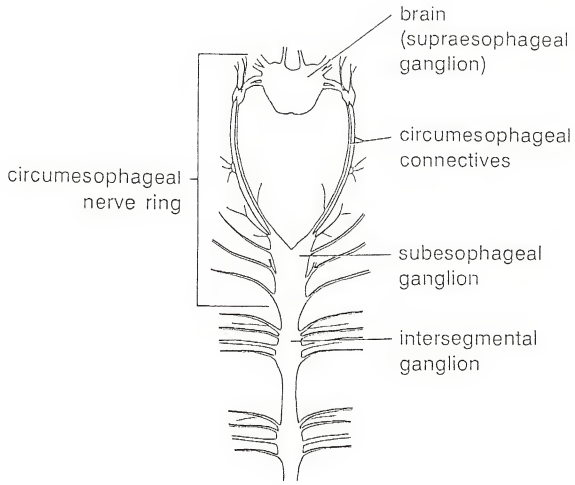


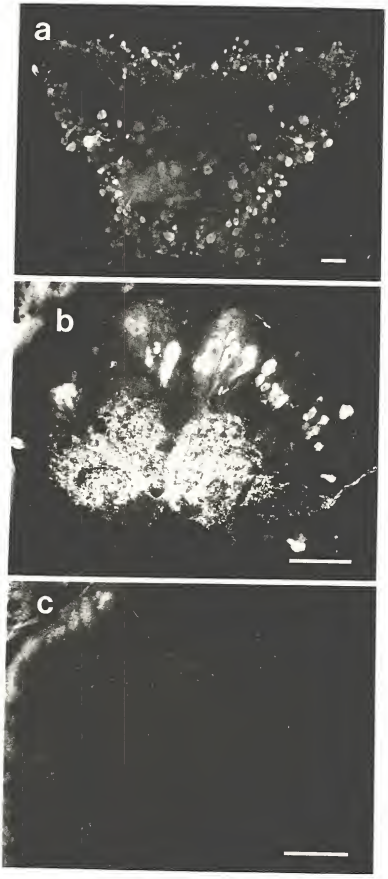
Figure 2-3. The central nervous system of *Nereis virens*. At the anterior end is the circumesophageal nerve ring composed of the brain (supraesophageal ganglion), the subesophageal ganglion, and the circumesophageal connectives. Posteriorly, the subesophageal ganglion is continuous with the ventral nerve cord which comprises the intersegmental ganglia and connectives.

In the brain (supraesophageal ganglion), both large and small cell bodies were stained, as were processes and varicosities in the neuropile (Figures 2-4a,b). The cell bodies were localized in the periphery of the ganglion and ranged in size from 10 to 70 μm. Immunoreactive varicosities and processes were seen in the connectives which lead to the subesophageal ganglion and some of the cephalic nerves.

Holmgren (1916) divided the nereid brain into 26 paired ganglionic cell groups or nuclei based upon their location in frontal sections. The first three nuclei densely packed mushroom shaped groups of cells referred to as Globuli I, II, III; the remaining 23 nuclei are non-mushroom shaped groups of cells and are designated by arabic numbers. When the outlines of these nuclei (taken from Holmgren) are superimposed upon the pattern of immunoreactive cell bodies in the nereid brain (Figure 2-5) the FMRFamidergic cells appear in Globuli I and III and nuclei 4, 5, 6, 7, 8, 10, 14, 15, 16, 17, 18, 19, 20, 22, and 23. FMRFamidergic cells may also be in nuclei 9 which is overlapped by nuclei 14, but frontal sections will be required to determine the exact nuclear location of the FMRFamidergic cell bodies.

Many immunoreactive cell bodies were present in the subesophageal ganglion (Figures 2-6). Also stained were the processes and varicosities in the ganglionic neuropile

Figure 2-4. Photomicrographs of FMRFamide immunoreactivity in the brain (supraesophageal ganglion) of *Nereis virens*. a. The ir-FMRFamide cell bodies and varicosities present in a wholemounted brain. Scale bar: 100 μm. b. A cross section of the brain showing immunoreactivity in both the cell bodies and neuropile. Scale bar: 100 μm. c. A cross section of the brain stained with preabsorbed FMRFamide antiserum. The fluorescence seen the upper left corner of 2-4b and c is non-specific fluorescence of the body wall covering the brain. Scale bar: 100 μm.


a
b
c

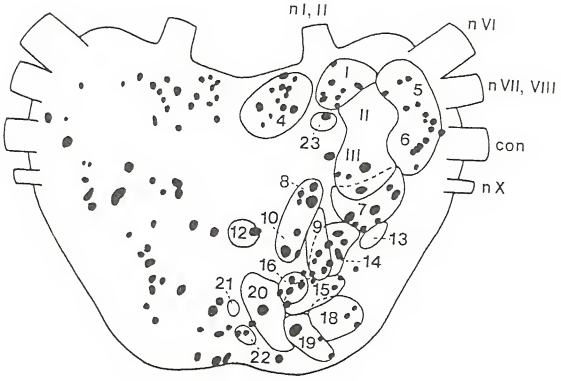
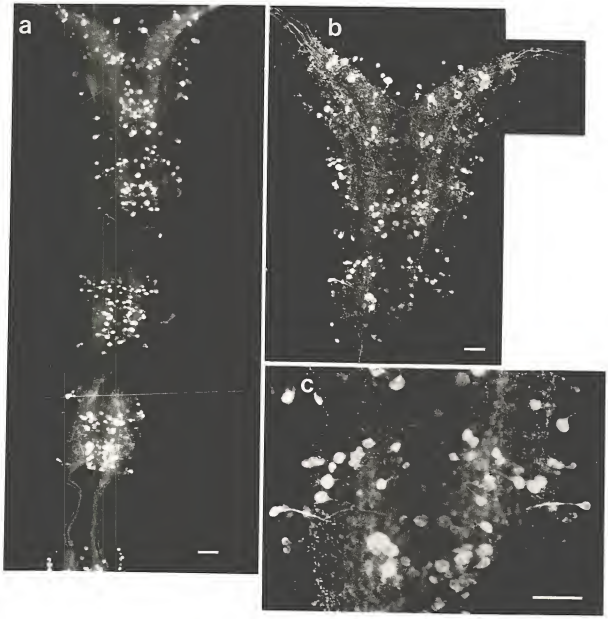


Figure 2-5. The positions of the 26 paired nuclei of the brain (supraesophageal ganglion) superimposed on the pattern of FMRFamide immunoreactive cell bodies. Only one of the symmetrically distributed, paired nuclei are shown on the right side of the ganglion. The first three nuclei, referred to as Globuli I, II, and III are marked in Roman numerals, while the remaining nuclei are marked in arabic numbers. The roots of some of the larger supraesophageal ganglionic nerves (nI, nII, nVI, nVII, nVIII, and nX) and the connective to the subesophageal ganglion (con) are shown. (The outline of the nuclei was adapted from Holmgren, 1916).

Figure 2-6. Photomicrographs of the FMRFamide immunoreactivity in the subesophageal ganglion and ventral nerve cord of *Nereis virens*. a. A wholemounted subesophageal ganglion and ventral nerve cord showing ir-FMRFamide cell bodies and varicosities. Scale bar: 100 µm. b. A close up of another wholemounted subesophageal ganglion showing FMRFamide immunoreactivity in the circumesophageal connectives. Scale bar: 100 µm. c. A close up of the last fused segment of the subesophageal ganglion in 2-6b, showing ir-FMRFamide cell bodies and processes. Scale bar: 100 µm.


a
b
c

and the connectives to the supraesophageal ganglion (Figure 2-6b). The cell bodies ranged in size from 10 to 60 μm.

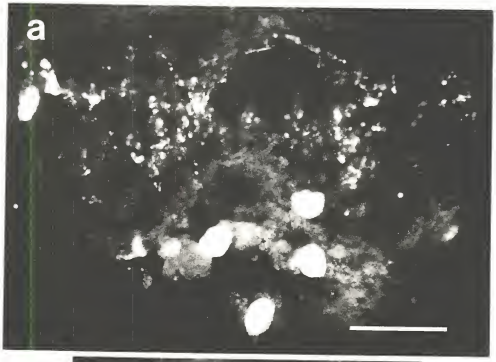
The intersegmental ganglia of the ventral nerve cord contained immunoreactive cell bodies and processes, both contralateral and longitudinal (Figures 2-6a, 2-7a,c). Cell body sizes ranged from 15 to 85 μm. Four major nerves leave from each ganglion (Smith, 1955, 1957) and FMRFamidergic fibers were present in each one. The interganglionic connectives also displayed many longitudinal FMRFamidergic processes.

Immunoreactive varicosities were also found in the gut (Figures 2-8a,b), parapodium (Figure 2-9a), body wall (Figure 2-9b), and palps (Figure 2-9c). The gut also contained a few cell bodies (Figure 2-8c).

Bioassay

The isolated nereid esophagus was spontaneously active, displaying a complex pattern of rhythmic contractions (Fig 2-10). Moderate to high doses of FMRFamide relaxed three of the four spontaneously active preparations studied, but the effects of low concentrations were difficult to ascertain because the spontaneous relaxations of the tissue occur unpredictably. Therefore, the esophagus was pre-contracted either by application of excitatory neurotransmitters, or transmural stimulation. Acetylcholine and serotonin caused small

Figure 2-7. Photomicrographs of immunoreactive FMRFamide in a wholemount and cross section of an intersegmental ganglion of *Nereis virens*. a. A cross section of an intersegmental ganglion showing the presence of FMRFamide in cell bodies and varicosities in the neuropile. Scale bar: 100 μm. b. A wholemount of an intersegmental ganglion showing FMRFamide immunoreactive processes projecting contralaterally through the ganglion. Scale bar: 100 μm.


a

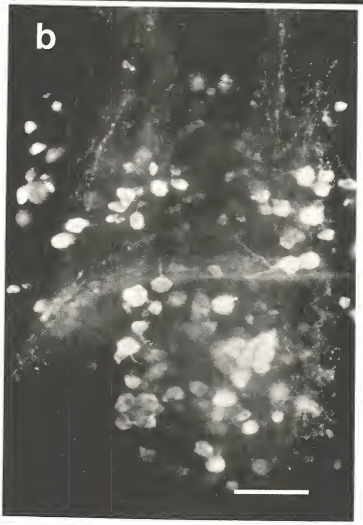

b

Figure 2-8. Photomicrographs of FMRFamide immunoreactivity in the gut of *Nereis virens*. a. A sagittal section of the esophagus showing FMRFamide immunoreactive varicosities. Scale bar: 50 μm. b. A cross section of the gut showing ir-FMRFamide in varicosities along the muscular layer. The arrows point out some of the varicosities in the in the nerve fiber. Scale bar: 50 μm. c. FMRFamide immunoreactive cell bodies in the same cross section of the gut as in 2-8b. The arrows are pointing to these cell bodies. Scale bar: 50 μm.

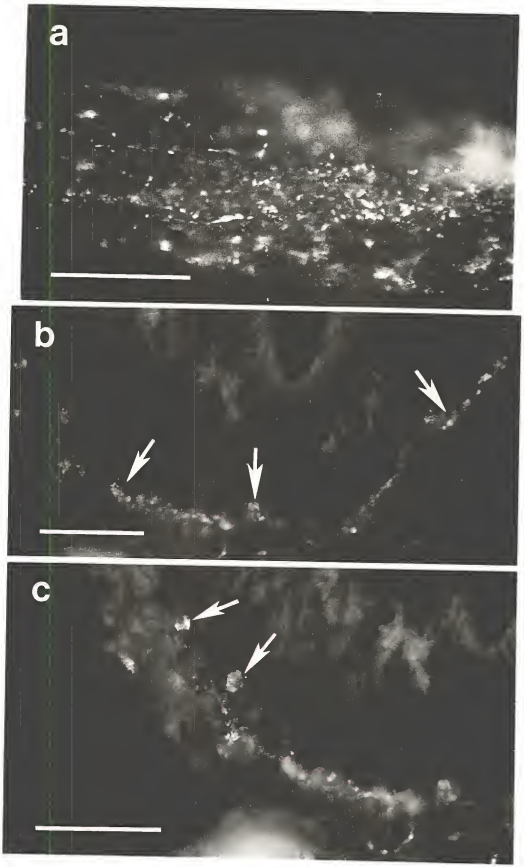

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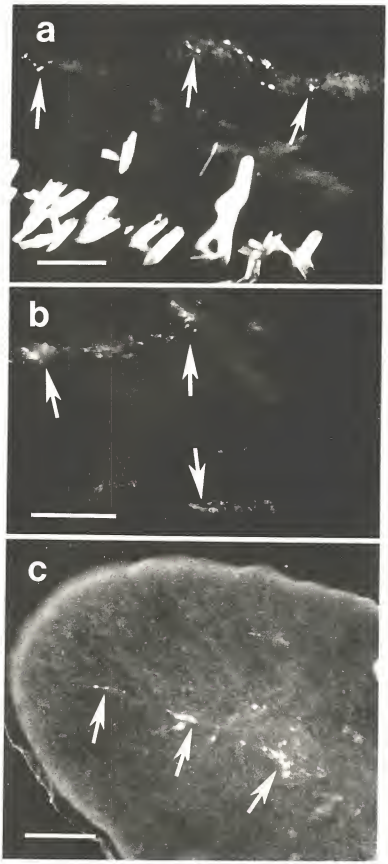
Figure 2-9. Photomicrographs of FMRFamide immunoreactive varicosities in the peripheral tissue of *Nereis virens*. a. Ir-FMRFamide varicosities in a cross section of a parapodium. Some of the varicosities along the path of the fiber are indicated by the three arrows. The brightly fluorescing objects below the nerve fiber are setae. Scale bar: 50 µm. b. FMRFamide immunoreactive varicosities in a cross section of the body wall. Some of the varicosities in the two fibers shown in this photo are indicated by the three arrows. Scale bar: 50 µm. c. FMRFamide immunoreactive varicosities in a cross section of a cephalic palp. Some of the varicosities along the nerve fiber are pointed to by the three arrows). Scale bar: 50 µm.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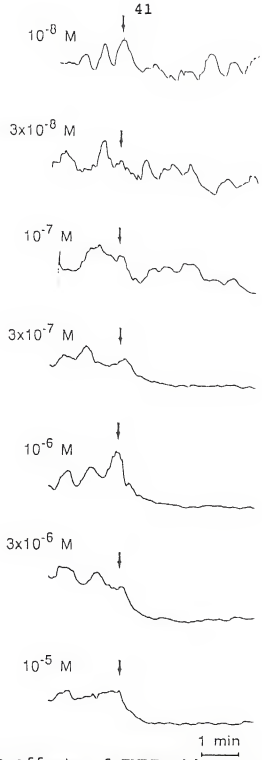


Figure 2-10. The effects of FMRFamide on the isolated, spontaneously active esophagus of *Nereis virens*. The down arrow indicates that FMRFamide was added to the tissue bath. The downward movement of the trace indicates a relaxation of the esophagus, while an upward movement indicates a contraction.

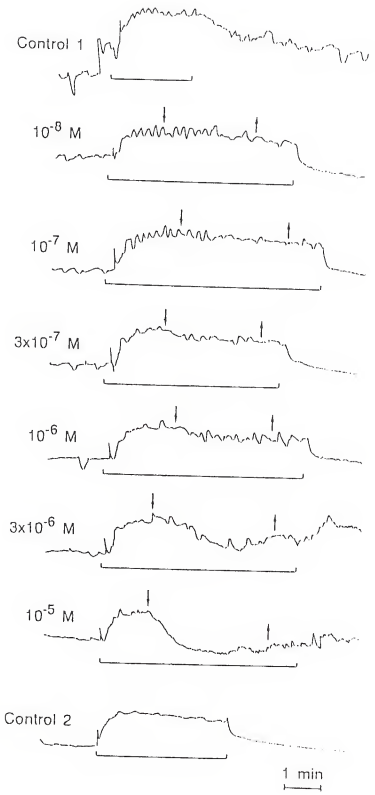
transient esophageal contractions that were not useful for the purposes of the bioassay (data not shown). But transmural electrical stimulation (2 Hz, 1 ms, 20 V) caused a more tonic increase in muscle tone, and was therefore used to contract the esophagus in the remaining experiments, although spontaneous relaxation still occurred in three of the eight preparations examined.

In both the spontaneously active and electrically stimulated preparations, FMRFamide relaxed the muscle tone with a threshold between 30 and 300 nM (Figures 2-10 and 2-11). The slope of the initial phase of relaxation of the electrically stimulated muscle increased in a dose-dependent manner (Figure 2-12) and the EC_{50} was 1.55±0.60 μM (N=5). Addition of neither the serotonin antagonist methysergide (UML) nor the acetylcholine antagonist benzoquinonium modified the effects of the peptide (Figure 2-13).

Discussion

The tetrapeptide FMRFamide has now been identified unequivocally in a non-molluscan species. This is the first time that exactly the same neuropeptide sequence has been identified in two different invertebrate phyla. The levels of FMRFamide in *Nereis* and molluscs are, however, quite different: i.e., 100-600 fmol per worm *versus* more than 10 pmol per animal in the gastropod mollusc *Aplysia brasiliana* (Lehman et al., 1984), a difference of 20-100

Figure 2-11. The effects of FMRFamide on the isolated, electrically stimulated esophagus of *Nereis virens*. The line under the trace indicates the duration of the electrical stimulation (2 Hz, 1 ms, 20 V). The down arrow indicates the addition of FMRFamide to the tissue bath. The up arrow indicates the washing of the tissue. Controls, electrical stimulation alone, were performed before and after the series of FMRFamide doses were given.


Control 1
10-8 M
10-7 M
3x10-7 M
10-6 M
3x10-6 M
10-5 M
Control 2
1 min

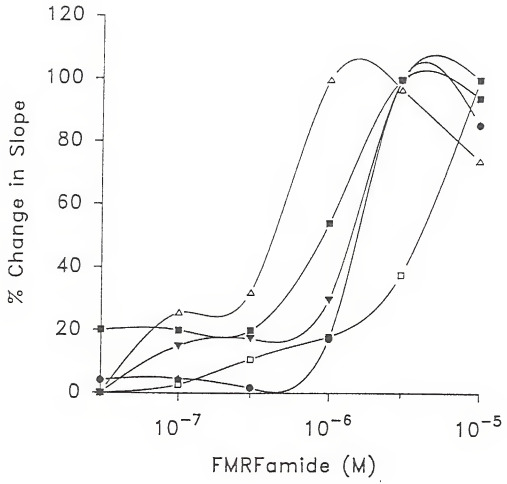


Figure 2-12. Dose-response curves of five different *Nereis* showing the effects of FMRFamide on the rate of relaxation of each electrically-stimulated esophagus. The slope of the initial relaxation of the esophagus was determined for each dose of peptide and then normalized to the maximum slope attained in that preparation. All points are adjusted to the maximal rate of relaxation for each animal. Each symbol represents one animal.

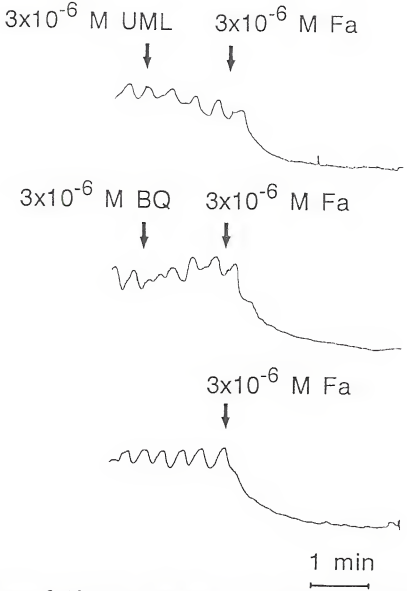


Figure 2-13. The effects of methysergide (UML) and benzoquinonium (BQ) on FMRFamide relaxation of the spontaneously active esophagus of *Nereis virens*. The down arrows indicate the addition of the antagonists and FMRFamide. The downward movement of the trace indicates a relaxation of the esophagus, while an upward movement indicates a contraction.

fold. This difference may be real, or it may reflect the difference in animal size, since the values were not normalized to the weight of tissue or the level of protein. Another phyletic difference is that FLRFamide, though present in all molluscs (Price and Greenberg, 1989), was not detected in the worms. Yet the presence of this peptide (or one or more other analogs) in Nereis cannot be ruled out; as in *Aplysia*, it may be present at very low levels relative to FMRFamide (Price and Greenberg, 1989).

All of the known genes encoding FaRP precursors contain two or more different peptides, and for at least one of these analogs, multiple copies are encoded (Schaefer et al., 1985; Taussig and Scheller, 1986; Nambu et al., 1988; Schneider and Taghert, 1988; Linacre et al., 1989; Lutz et al., 1990; Taghert and Schneider, 1990). Therefore, the *Nereis* gene probably also encodes for FMRFamide and one or more analogs. If the ratio of FLRFamide (or any other analog) to FMRFamide is 1/28-1/5 in *Nereis*, as it is in molluscs, then the level of this peptide would be 3.5-120 fmol per worm and it would probably not be seen. In summary, then other analogs in the precursor might not be detectable.

Phylogenetically the annelids, molluscs, and arthropods have many characteristics which suggest that they have a common evolutionary lineage. The data from

Nereis are consistent with this hypothesis and suggest that the FMRFamide tetrapeptide gene may have arisen in the ancestry of that lineage.

The presence of ir-FMRFamide in the cell bodies and processes of the circumesophageal nerve ring and ventral nerve cord agrees with the preliminary data of Porchet and Dhainaut-Courtois (1988). They showed that immunoreactive cell bodies occurred in the supraesophageal ganglion of *Nereis diversicolor* in nuclei 7, 10, and 13.

Many of the 26 nuclei in the nereid brain contain both secretory and non-secretory cell types, based upon the selective staining of secretory cells with paraldehyde fuschin (Golding, 1967; Engelhardt et al., 1982). Staining the same nereid tissue sections with both paraldehyde fuschin and antibodies to the vertebrate peptide cholecystokinin (CCK), Engelhardt et al. (1982) showed that CCK immunoreactivity was associated with both cell types, and suggested that a CCK-like peptide might function as both a neurotransmitter and neurohormone. FMRFamidergic cells appear to be in some of the same nuclei, so they may also have both a neurohumoral and a neurotransmitter function, although further investigation would be required to confirm this.

The intersegmental ganglia each contain about 200 neurons in a crescent-shaped cortex along the ventral and lateral edges of the nerve cord (Smith, 1957). Of these

200 cells, only 7 pairs are motor neurons; the remainder are interneurons with their processes in the ganglionic neuropile. Since these ganglia contain at least 40 immunoreactive cell bodies, some of them must be FMRFamide containing interneurons.

Motor neurons in the intersegmental ganglia may also be FMRFamidergic. To confirm this, however, the entire neuron must be traced from its cell body in the cortex, along its axon in the neuropile, to the ganglionic nerve through which the axon exits, and to the target organ. No cells could be so traced, but FMRFamidergic processes do cross the ganglia contralaterally, as some motor neurons have been shown to do (Smith, 1955; 1957). Some immunoreactive processes do appear in each of the four ganglionic nerves, but these could either be sensory or motor fibers. Nevertheless, the occurrence of FMRFamidergic varicosities in the body wall muscles strongly suggests that at least some of these fibers are motor in function.

All of the FMRFamide extracted in acetone came from the first 3 cm of the animal and the levels observed were consistent with those estimated from the immunohistochemical data. When the remaining 17 cm of the animal were extracted, however, no peptide was found, suggesting that the level of peptide declined toward the more posterior ganglia. Indeed, this distribution pattern

appears to occur in two other annelids; i.e., the number of ganglionic immunoreactive cells decreased toward the tail in both the leech (Kuhlman et al., 1985a; Calabrese and Norris, 1990) and the earthworm (Fujii, 1989).

The anatomical and physiological data indicate that FMRFamide is involved in the control of nereid intestinal motility. FMRFamidergic varicosities and cell bodies were observed in the gut. The polychaete somatogastric nervous system is composed of a network of ganglionic and sensory neurons, including their processes in the wall of the gut (Whitear, 1953). The functions of the cells containing the peptide is unclear. In the sedentary, burrow-dwelling polychaete, *Arenicola*, the esophagus is innervated by the somatogastric nervous system, and when it is isolated from the rest of the worm, the tissue contracts with a distinct rhythm suggesting the presence of an endogenous pacemaker (Wells, 1937). In the nereid esophagus, FMRFamide at low concentrations inhibits the rhythmicity of some preparations, suggesting that the peptide may be involved in modulation of the pacemaker. The failure of serotonergic and cholinergic antagonists to inhibit the response to FMRFamide suggests that the peptide is not acting on the esophagus by releasing serotonin or acetylcholine from nerve terminals. The involvement of FMRFamide in intestinal motility may be a general function in annelids since FMRFamidergic fibers are present in the

gut of the earthworm *Eisenia foetida* (Fujii et al., 1989).

In the closely related phylum Mollusca, FMRFamide inhibits the feeding and gastric motility of gastropods by acting within the CNS (central nervous system) as well as the musculature of the alimentary tract (Krajniak et al., 1989). The involvement of FMRFamide in feeding at the level of the CNS in annelids remains to be studied.

The localization of FMRFamidergic varicosities in the muscles of the body wall could suggest that the peptide plays a role in body movement. But it could further the proposed role for FMRFamide in the intestinal motility. In *Nereis*, the musculature of the body wall (as well as that of the gut) is involved in the propulsion of food through the animal. The septal muscles dilate the intestine and cooperate with the suspensory muscles and body wall muscles in the complicated intestinal movements (Michel and Devillez, 1978). In any event, FMRFamide immunoreactivity has been found in the body walls of *Hirudo medicinalis* (Kuhlman et al., 1985a), *Sabellastarte magnifica* (Diaz-Miranda et al., 1989), and *Eisenia foetida* (Fujii et al., 1989). The effects of FMRFamide on the body wall of *Nereis* were not examined because its attachment to the intestine via the septa between each body segment precludes a clean, isolated body wall preparation. In *S. magnifica*, however, an isolated body wall preparation has been made, and FMRFamide relaxes a

dopamine-induced contraction of musculature (Diaz-Miranda et al., 1989).

FMRFamide immunoreactivity was also observed in the palps and in the supraesophageal ganglionic nerve roots that supply them. The palps are sensory structures, but the FMRFamidergic varicosities within them could be associated with motor neurons or interneurons, as well as sensory neurons.

CHAPTER 3

THE FMRFamide-RELATED PEPTIDE OF *CALLINECTES SAPIDUS*

Introduction

The occurrence of FMRFamide-related peptides (FaRPs) in the superphylum Arthropoda is now firmly established. Five peptides, all N-terminal extensions of the tetrapeptide core (FXRFamide, where X is Leu, Met, or Ile) have been isolated and sequenced. Another 12 peptides are encoded in the genes of two species of *Drosophila*.

The arthropodan FaRPs can be divided further into three structurally distinct groups, two in insects and another in crustaceans and insects (Table 3-1). Group 1 includes two insect peptides, one from the cockroach *Leucophaea maderae* (Holman et al., 1986) and another from the locust *Schistocerca gregaria* (Robb et al., 1989); both contain the common C-terminal core, Val-Phe-Leu-Arg-Phe-NH_2. Group 2 includes peptides from two species of fruit fly, *Drosophila melanogaster* and *D. virilis* (Nambu et al., 1988; Taghert and Schneider, 1990) which share the C-terminal sequence Asp-Phe-X-Arg-Phe-NH_2 (where X is either Met or Val). Group 3 comprises peptides from the two species of *Drosophila* and the crustacean *Homarus*

TABLE 3-1. ARTHROPODAN FMRFamide-RELATED PEPTIDES.

Nuclear Sub-Family Group	Sequence	Species
1	pGlu-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2	*L. maderae*
	Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2	*S. gregaria*
2	Asp-Pro-Lys-Gln-Asp-Phe-Met-Arg-Phe-NH_2*	*D. melanogaster/ D. virilis*
	Thr-Pro-Ala-Glu-Asp-Phe-Met-Arg-Phe-NH_2	
	Ser-Pro-Lys-Gln-Asp-Phe-Met-Arg-Phe-NH_2	
	Ser-Arg-Pro-Gln-Asp-Phe-Val-Arg-Phe-NH_2	
	Ala-Pro-Pro-Ser-Asp-Phe-Met-Arg-Phe-NH_2	
	Ala-Pro-Ser-Asp-Phe-Met-Arg-Phe-NH_2	
3	Ser-Asp-Asn-Phe-Met-Arg-Phe-NH_2	D. melanogaster/
	Pro-Asp-Asn-Phe-Met-Arg-Phe-NH_2	D. virilis
	Met-Asp-Ser-Asn-Phe-Met-Arg-Phe-NH_2	
	Met-Asp-Ser-Asn-Phe-Ile-Arg-Phe-NH_2	
	Ser-Val-Gln-Asp-Asn-Phe-Met-His-Phe-NH_2	
	Ser-Leu-Gln-Asp-Asn-Phe-Met-His-Phe-NH_2	
	Ser-Asp-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	*H. americanus*
	Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	

*The only *Drosophila* gene product which has also been isolated and sequenced as a peptide. All other *Drosophila* peptides listed are predicted by the gene.

americanus (Trimmer et al., 1987); they contain the common C-terminal sequence Asn-Phe-X-Arg-Phe-NH_2 (where X is Met, Leu, or Ile).

The arthropodan FaRPs have many biological effects. In insects, the peptides of group 2 potentiate the twitch of the neurally evoked locust extensor tibiae muscle (Cuthbert and Evans, 1989) and inhibit the contractions of cockroach hindgut (Holman et al., 1986) and the locust heart (Cuthbert and Evans, 1989; Robb et al, 1989). The group 3 FaRPs stimulate the lobster heart (Trimmer et al.,1987), modulate crayfish neuromuscular synapses (Mercier et al., 1989), and stimulate the crab somatogastric ganglion (Weimann and Marder, 1989). Hooper and Marder (1984) showed that FMRFamide, a non-arthropodan FaRP, also stimulated the somatogastric ganglion.

Four different insects have been examined and FaRPs representative of all three arthropodan peptide groups have been found. But only one crustacean has been studied, and only peptides from group 3 were found (Trimmer et al., 1987). I therefore chose to study a second decapod crustacean to determine the sequence variability within this order. I hoped further to determine whether crustaceans would also contain peptides with the characteristics of groups 1 or 2.

The lobster FaRPs were isolated from the pericardial

organs (Trimmer et al., 1987), and the highest concentrations of ir-FMRFamide throughout the entire nervous system were in these organs (Kobierski et al., 1987). The cells innervating the pericardial organs have their somas in the thoracic ganglion; the organs themselves are neurohemal release sites that lie in the venous cavity surrounding the crustacean heart (Cooke and Sullivan, 1982) (Figure 3-1). Hemolymph flows through the gossamer structures of the pericardial organs and into the heart, making them the perfect release point for cardioactive neurohormones. Many other cardioexcitatory substances are found in this tissue, including such peptides as, proctolin (Sullivan, 1979) and crustacean cardioexcitatory peptide (CCAP)(Stangier et al., 1987), and the classic neurotransmitters serotonin (Beltz and Kravitz, 1983), octopamine (Livingstone et al., 1981), and dopamine (Cooke and Sullivan, 1982).

Therefore, I have isolated and sequenced peptides from the pericardial organs and thoracic ganglia of the blue crab *Callinectes sapidus*, which is commercially available in large numbers. Furthermore, since Trimmer et al. (1987) indicated that the *Homarus* FaRPs were cardioexcitatory, the structure-activity relationship of the blue crab cardiac receptor was examined.

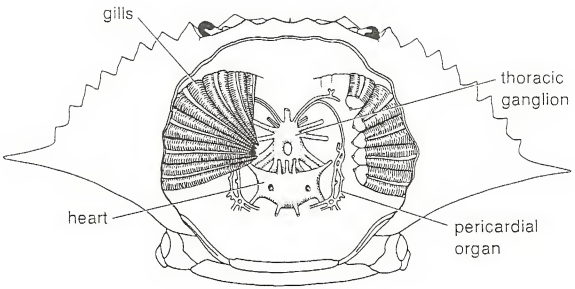


Figure 3-1. A schematic of the blue crab *Callinectes sapidus*. The drawing is a dorsal view of an animal with part of its dorsal carapace removed showing the placement of the heart, thoracic ganglion, pericardial organs and gills (redrawn after Maynard, 1961).

Methods

Animals

Blue crabs, *Callinectes sapidus*, were purchased from a commercial supplier and maintained in flowing, filtered seawater for at least one week prior to experimentation. The animals were fed squid at least twice a week.

Peptide Extraction, Purification, and Sequencing

The pericardial organs or thoracic ganglia were dissected from the crabs and placed in 10 volumes of acetone. The mixture was maintained at -20° C and kept until the tissues from 100 to 500 animals had accumulated. Once the desired amount of tissue was acquired, the acetone was decanted, and the extract was processed as described in Chapter 2.

The processed aqueous residue was loaded onto either a Waters Novapak C-18 reverse-phase HPLC column (3.9 x 150 mm) with a flow rate of 2 ml/min, or a Brownlee Prep 10 Aquapore Octyl C-8 reverse-phase HPLC column (10 x 100 mm) with a flow rate of 4 ml/min. The columns were eluted with a gradient of ACN (0 to 40% in 30 min) containing 0.1% TFA. Fractions were collected every 30 s, and a 2 µl aliquot from each was assayed in an RIA for FMRFamide; the assay employed both the S253 and Q2 antisera (details in Chapter 2).

The fractions from the first separation were further purified on either the Waters Novapak C-18 HPLC column or

a Brownlee RP-300 Aquapore Octyl reverse-phase HPLC column (2.1 x 220 mm) with a flow rate of 0.5ml/min. A second solvent system, of either isopropanol with 0.1% TFA (10 to 40% in 20 min) or ACN with 0.1% heptafluorobutyric acid (HFBA) (16 to 32% in 20 min), was used in alternation with the ACN/TFA system (16 to 32% in 20 min) on these columns until each immunoreactive peak was coincident with a single peak at 210 nm absorbance.

Material form the peaks of the purified peptide were submitted for microsequencing to the Protein Chemistry Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research, and for FAB-mass spectroscopy to Dr. T.D. Lee, Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California.

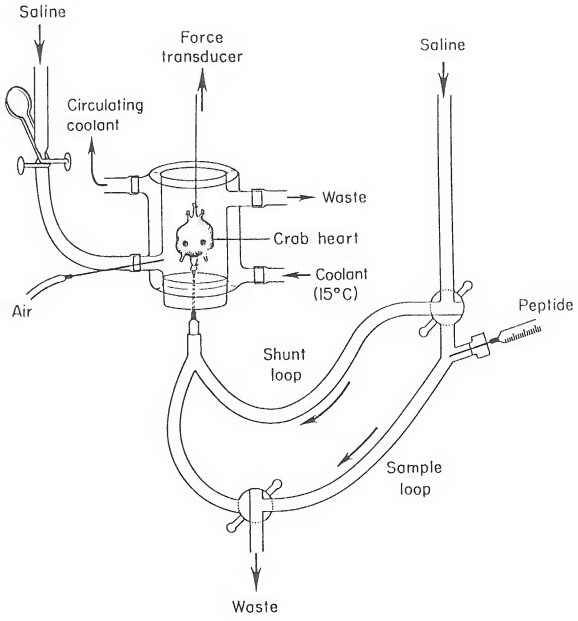
Bioassay

A crab was removed from the holding tank and placed in ice for at least 30 min. Once anesthetized, the dorsal carapace was opened and the heart removed. With the aid of dissecting microscope, I secured a cannula was secured in the sternal artery with thread. The cannula was connected with the reservoir of crab saline suspended above a level of the heart. Most of the other arteries were ligated, but the posterior aortas were left patent so that the heart could empty during systole. The heart was suspended in a temperature controlled tissue bath (Figure

3-2) maintained at 15° C, and perfused with a crustacean saline at a constant pressure of 42 cm H_2O (4.2 kPa) and a flow rate of 3 ml/min (Fig. 3-2). The saline contained 458.6 mM sodium chloride, 13.6 mM potassium chloride, 13.4 mM calcium chloride, 13.6 mM magnesium chloride, 1.4 mM sodium sulfate, 3 mM HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), adjusted to pH 7.4 with sodium hydroxide (Mulloney and Selverston, 1974). Contractions of the heart were recorded with a Grass force/displacement transducer attached to the anterior aorta and monitored on a Grass recorder.

All peptides were diluted to the desired concentration in crab saline (final volume of 0.5 ml) and were introduced into the heart via the sample loop. Addition of peptide by this method did not disturb the flow of saline to the heart. Prior to the addition of any peptides, 0.5 ml of saline were loaded into the sample loop and injected to ensure that the mechanical components of the system had no effect on the heart. Each heart was exposed to a test peptide and the standard, GYNRSFLRFamide. Starting with the lowest concentrations, samples of these two were added alternately. The sample loop was replaced after each peptide, and saline was injected prior to the next peptide to control the

Figure 3-2. The perfused *Callinectes* heart isolated in a bioassay apparatus. The crab heart is suspended in a 15° C tissue bath and is connected to the saline reservoir by a cannula through its thoracic artery. The saline reservoir is a Mariotte bottle which kept the perfusion pressure constant at 42 cm H_2O (4.2 kPa) and the flow rate at 3 ml/min. The cannula is connected to both the sample loop and the shunt loop which are regulated by the two valves. While the shunt loop is open to the heart, the sample loop which is open to the waste reservoir can be filled with peptide without disturbing the perfusion. The sample loop is then closed off to the waste line and the shunt loop is closed off to the heart without interrupting saline flow rate or pressure. The perfusate passes through the heart and into the inner chamber of the tissue bath to help buoyantly support the heart and maintain the temperature. The bottom of the inner chamber is connected via rubber tubing to the same Mariotte bottle saline reservoir so that the entire chamber can be flushed. A needle attached to a source of compressed air is inserted into this rubber tubing to oxygenate the tissue. Excess waste water flows out of the top of the chamber to a waste reservoir. The anterior aorta is connected to the force/displacement transducer which allows the contractions to be recorded.


Saline
Force transducer
Saline
Circulating coolant
Waste
Crab heart
Coolant (15°C)
Air
Peptide
Shunt loop
Sample loop
Waste

possibility that some of the previous dose was still in the line.

The peptides always affected heart rate and the change in frequency was therefore the parameter used to quantify the effects of each peptide. When comparing a peptide to GYNRSFLRFamide, doses of each peptide were used to construct the linear portions of their curves around their EC_{50}s. The relative potency of the test peptide to GYNRSFLRFamide was determined by calculating the difference between the two lines at the EC_{50} (Tallarida and Murray, 1981). In some experiments full dose-response curves were generated, and the maximal effect of the test peptide was compared with that of GYNRSFLRFamide, so that the relative maximal effect (efficacy) could be determined.

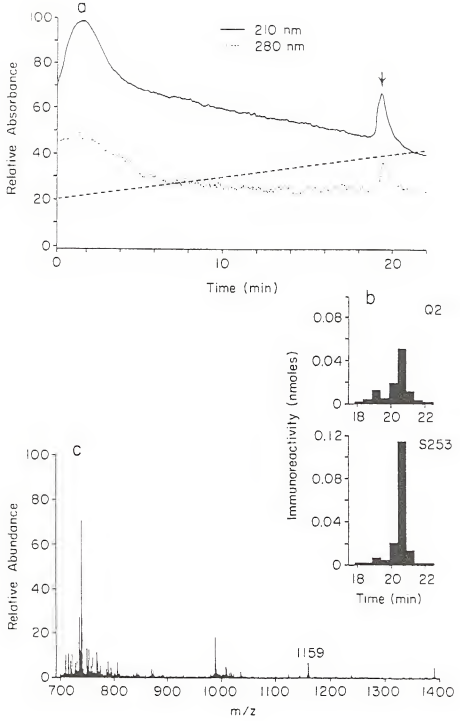
Synthetic peptides were either purchased from Peninsula, Sigma, or synthesized by Dr. Ben Dunn and Alicia Alvarez of the Protein Chemistry Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research.

Results

Peptide Extraction, Purification, and Sequencing

In one experiment with 512 animals, a peak of immunoreactivity with absorbance at both 210 nm and 280 nm was purified (Figure 3-3). When analyzed by FAB-mass spectrometry, it yielded a major ion at m/z 1159. Thus,

Figure 3-3. The HPLC purification and FAB-mass spectrum of the *Callinectes* FaRP isolated from 512 animals. a. The UV absorbance at 210 nm (solid line) and 280 nm (dotted line) of the final HPLC purification step. The dashed line shows the gradient of ACN with 0.1% HFBA. b. The amount of FaRP determined by RIA with antisera Q2 and S253 in the UV absorbance peak in 3-3a. The lag between the peaks of UV absorbance and immunoreactivity reflects the dead space between the detector and the fraction collector. c. Mass spectrometry of the immunoreactive fraction in 3-3b, showing a normal (magnetic sector scan) positive-ion FAB- mass spectrum for the peptide. The other ions in the spectrum are due to sample matrix.

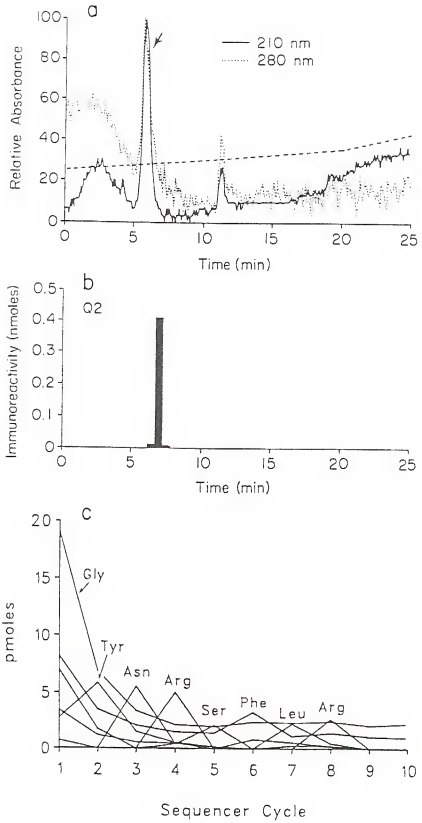

a
210 nm
280 nm
Relative Absorbance
Time (min)
b
Q2
S253
Immunoreactivity (nmoles)
Time (min)
c
Relative Abundance
1159
m/z

the peak contained an immunoreactive peptide with a molecular weight of 1158.

In another experiment (200 animals) a similar immunoreactive peak with absorbance at 210 and 280 nm was observed (Figure 3-4). It eluted off the column at the same time as the peak in the previous experiment when the same HPLC gradients and buffer systems were used (data not shown). (Note that different buffer systems and gradients were used in the experiments illustrated in Figures 3-3a and 3-4a, so the peaks elute at different times.) When the purified peak was sequenced, the identity of the first amino acid was unclear because the levels of all amino acids in this cycle were high, including that of glycine, a common contaminant (Figure 3-4a). The remainder of the sequence was determined and indicated a peptide with the partial sequence X-Tyr-Asn-Ser-Phe-Leu-Arg, where X represents an unknown amino acid in the first position (Fig 3-4a). This incomplete sequence also lacked a C-terminal phenylalanine-amide which is seen in all other FaRPs. The calculated molecular weight of this fragment was 955, and the difference between it and the observed molecular weight of 1158 (taken from the peak in the previous experiment) was 203.

Only two amino acid combinations, plus an amide, could account for this difference: (cysteine, threonine) and (glycine, phenylalanine). Cysteine was not present in

Figure 3-4. The HPLC purification and sequence of the *Callinectes* FaRP from 200 animals. a. The UV absorbance at 210 nm (solid line) and 280 nm (dotted line) of the final HPLC purification step of the FaRP. The dashed line shows the gradient of ACN with 0.1% TFA. b. The amount of FaRP determined by RIA with antiserum Q2 in the UV peak of 3-4a. The lag between the peaks of UV absorbance and immunoreactivity reflects the dead space between the detector and the fraction collector. c. The microsequence of the immunoreactive fraction in 3-4b, showing the changes in glycine, tyrosine, asparagine, arginine, serine, phenylalanine, and leucine in each cycle.


a
210 nm
280 nm
Relative Absorbance
Time (min)
b
Q2
Immunoreactivity (nmoles)
Time (min)
c
Gly
Tyr
Asn
Arg
Ser
Phe
Leu
Arg
pmoles
Sequencer Cycle

any of the sequencer cycles, so the missing residues must be glycyl and phenylalanyl. Thus the peptide sequence predicted was Gly-Tyr-Asn-Arg-Ser-Phe-Leu-Arg-Phe-NH_2 (GYNRSFLRFamide). The presence of the C-terminal Phe-NH_2 was further indicated by the binding of the peptide to antiserum S253, which requires an amidated phenylalanine in that position (Price, 1983; Price 1987; Greenberg et al., 1988). Furthermore, the sequence Phe-Leu-Arg-Phe was found in yet another purification of pericardial organs, and could be a breakdown product of GYNRSFLRFamide (Table 3-1). The predicted weight of the protonated peptide is 1158.6, which is very close to the observed ion of 1159. Synthetic GYNRSFLRFamide has the same elution time on the HPLC as the native peptide, when either the ACN/TFA or the ACN/HFBA buffer systems are used.

The levels of acetone-extracted peptide are very low in these animals. In the 200 animal experiment, the initial yield of peptide estimated by RIA in the GYNRSFLRFamide peak is 2.67 nmol (13.4 pmol/animal). The purified peak yielded only 410 pmol by RIA with antiserum Q2 (2 pmol/animal), 8 pmoles by UV absorbance (40 fmol/animal), and 7 pmol by microsequencing (35 fmol/animal). In the 512 animal experiment, the initial peak was 3.4 nmoles (6.6 pmol/animal) by RIA. The purified peak contained 113 pmol (50 pmol with Q2) by RIA (from 98 to 220 fmol/animal) and 12 pmol by UV absorbance

(23 fmol/animal). One reason for the such a small yield of peptide may be the method of extraction. Trimmer et al. (1987) reported that acidified methanol extracted more pericardial organ peptides than pure acetone. However, acidified methanol extracted more impurities (indicated by the increased amount of UV absorbance during the initial HPLC purification step) and required more purification steps with associated losses in peptide.

In addition to the experiment described above, multiple batches of pericardial organs and thoracic ganglia were extracted, purified, and sequenced. In most cases, the initial separation of the extract yielded at least two peaks of FMRFamide immunoreactivity. Upon completion of the HPLC purification, partial amino acid sequences were observed in all instances (Table 3-2). The array of sequences suggests that the crab contains several different analogs of GYNRSFLRFamide; and that the sequences Phe-Leu-Arg-Phe, Asn-Phe-Leu-Arg-Phe, and Ser-Phe-Leu-Arg are probably fragments of these larger native peptides.

Structure-Activity Relationship of the Cardiac Receptor

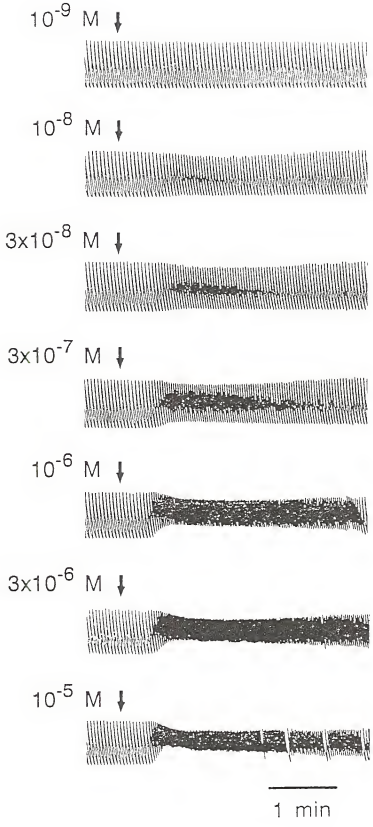
The isolated heart of *Callinectes sapidus* beat spontaneously in the tissue bath at 15° C. The average resting heart rate prior to any peptide administration was 26.6±1.0 beats per minute (beats/min) (N=132). The average heart rate of intact animals at 15° C is 62.3±10.0

TABLE 3-2. PARTIAL SEQUENCES OF THE *CALLINECTES* FaRP.

TISSUE SOURCE	PEPTIDE SEQUENCE*
Pericardial organ	X -Tyr-Asn-Arg-Ser-Phe-Leu-Arg
Pericardial organ	Ser-Phe-Leu-Arg
Pericardial organ	X - X -His-Lys-Asn-Tyr-Leu-Arg-Phe
Pericardial organ	X - X -Met-Asn-Phe-Leu-Arg
Pericardial organ	Gly-Asn-Arg-Asn-Phe-Leu-Arg
Pericardial organ	X -Arg-Asn-Phe-Leu-Arg
Pericardial organ	Arg-Asn-Phe-Leu-Arg
Pericardial organ	X - X -Asn-Phe-Leu-Arg
Pericardial organ	Asn-Phe-Leu-Arg-Phe
Pericardial organ/Thoracic ganglion	Phe-Leu-Arg-Phe

* X represents an unknown amino acid in the sequence.

Figure 3-5. The effects of increasing doses of GYNRSFLRFamide on the isolated heart of the blue crab *Callinectes sapidus*. The figure shows the effects of increasing doses (indicated on the left) of GYNRSFLRFamide on heart rate and contraction amplitude. The down arrow indicates when the sample loop was opened to the heart.


10^{-9} M
10^{-8} M
$3x10^{-8}$ M
$3x10^{-7}$ M
10^{-6} M
$3x10^{-6}$ M
10^{-5} M
1 min

beats/min (N=6) (deFur and Mangum, 1979). Such differences are to be expected, since both heart rate and muscle tone vary with perfusion pressure (Maynard, 1960; Kuramoto and Ebara, 1984; 1988), and the conditions under which the heart is isolated do not approximate those in the intact animal.

GYNRSFLRFamide excited the heart in a dose dependent manner (Figure 3-5). The excitation always consisted of an increase in heart rate. Contraction amplitude and tone either increased, decreased, or remained unchanged. Because of this inconsistency, heart rate was chosen as the measured variable. GYNRSFLRFamide had a threshold of between 10 and 30 nM and an EC_{50} of 323±62 nM (N = 11) (Figure 3-6).

Sixteen analogs of GYNRSFLRFamide were tested on the isolated crab heart bioassay (Table 3-3). All of the C-terminally amidated FaRPs were cardioexcitatory, increasing heart rate and changing amplitude and tone in a manner similar to that of the native *Callinectes* peptide. The *Homarus* peptide TNRNFLRFamide, the most potent analog, was one order of magnitude more effective than GYNRSFLRFamide. The other known lobster peptide (SDRNFLRFamide) and three other synthetic N-terminally extended FaRPs (VNRNFLRFamide, LRNFLRFamide, and VNNFLRFamide) were roughly equipotent with the crab FaRP.

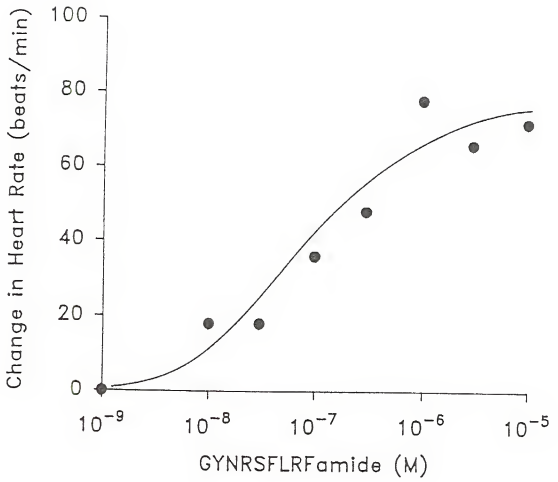


Figure 3-6. A dose response curve, showing the effects of GYNRSFLRFamide on the heart of *Callinectes sapidus*. The curve shows the change in heart rate plotted against the concentration of GYNRSFLRFamide injected into the heart. These data come from the experiment illustrated in Figure 3-5.

TABLE 3-3. RELATIVE POTENCIES OF SOME FaRPS ON THE ISOLATEDHEART OF THE BLUE CRAB *CALLINECTES SAPIDUS*.

PEPTIDE SEQUENCE	RELATIVE POTENCY[a]	N
1 2 3 4 5 6 7 8 9[+]		
Gly-Tyr-Asn-Arg-Ser-Phe-Leu-Arg-Phe-NH_2	1	
Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	22.4 ±13.6	16
Ser-Asp-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	4.96±0.53	11
Val-Asn-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	4.84±1.91	3
Leu-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	1.35±0.15	8
Val-Asn-Asn-Phe-Leu-Arg-Phe-NH_2	4.35±2.76	4
Arg-Asn-Phe-Leu-Arg-Phe-NH_2	0.17±0.02	7
Glu-Asn-Phe-Leu-Arg-Phe-NH_2	0.48±0.14	6
Gly-Asn-Phe-Leu-Arg-Phe-NH_2	0.39±0.09	6
pGlu-Asn-Phe-Ile-Arg-Phe-NH_2	0.22±0.03	2
Arg-Leu-Phe-Leu-Arg-Phe-NH_2	0.02±0.01	6
Arg-Gln-Phe-Leu-Arg-Phe-NH_2	0.09±0.05	6
Arg-Ser-Phe-Leu-Arg-Phe-NH_2	0.07±0.04	6
Asn-Phe-Leu-Arg-Phe-NH_2	0.30±0.01	7
Phe-Leu-Arg-Phe-NH_2	0.06±0.01	10
Phe-Met-Arg-Phe-NH_2	0.02±0.01	6
Phe-Leu-Arg-Phe-OH	No Response[*]	3

[a] ±Standard error.
[+] The numbers designate the amino acid positions in GYNRSFLRFamide for the discussion of amino acid substitutions.
[*] At concentrations up to 100 μM.

Since the lobster peptides and at least one partially sequenced *Callinectes* peptide (Table 3-2) have the sequence RNFLRFamide in common, this peptide and several different analogs with amino acid substitutions in either position 4 or 5 (as numbered in GYNRSFLRFamide) were examined, especially to study the importance of the arginine and the asparagine. When analogs with the sequence XNFLRFamide (where X is Arg, Gly, pGlu, or Glu) were used, they were about an order of magnitude less potent on the heart than GYNRSFLRFamide. The same was true for the pentapeptide NFLRFamide. When the sequence was changed to RXFLRFamide (where X is Leu or Gln), the potency decreased further relative to GYNRSFLRFamide. But note that the substitutions of glutamine and serine substitutions are not nearly so adverse as that of the leucine, which was expected since glutamine and serine are, like asparagine, hydrophilic. Similarly the core tetrapeptides, FLRFamide and its analog FMRFamide, were also about two orders of magnitude less potent than GYNRSFLRFamide. The unamidated tetrapeptide FLRF-OH had no biological activity at concentrations up to 100 μM. The relative efficacies of the analogs ranged from 1.25 for TNRNFLRFamide, to 0.80 for ENFLRFamide (Table 3-4), indicating that all the peptides in this study could reach approximately the same maximal response as GYNRSFLRFamide.

TABLE 3-4. RELATIVE EFFICACIES OF SOME FaRPS ON THE ISOLATED HEART OF THE BLUE CRAB *CALLINECTES SAPIDUS*.

PEPTIDE SEQUENCE	RELATIVE EFFICACY	N
Gly-Tyr-Asn-Arg-Ser-Phe-Leu-Arg-Phe-NH_2	1.00	
Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	1.25	1
Ser-Asp-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	1.19	3
Val-Asn-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	1.00	1
Leu-Arg-Asn-Phe-Leu-Arg-Phe-NH_2	0.98	2
Val-Asn-Asn-Phe-Leu-Arg-Phe-NH_2	1.00	1
Arg-Asn-Phe-Leu-Arg-Phe-NH_2	0.84	3
Glu-Asn-Phe-Leu-Arg-Phe-NH_2	0.80	3
Gly-Asn-Phe-Leu-Arg-Phe-NH_2	N.D.$^+$	0
pGlu-Asn-Phe-Ile-Arg-Phe-NH_2	N.D.$^+$	0
Arg-Leu-Phe-Leu-Arg-Phe-NH_2	0.84	3
Arg-Gln-Phe-Leu-Arg-Phe-NH_2	0.86	2
Arg-Ser-Phe-Leu-Arg-Phe-NH_2	0.89	2
Asn-Phe-Leu-Arg-Phe-NH_2	0.83	3
Phe-Leu-Arg-Phe-NH_2	0.85	4
Phe-Met-Arg-Phe-NH_2	0.86	2
Phe-Leu-Arg-Phe-OH	N.R.*	3

* No response at concentrations up to 100 μM.
$^+$ Not determined.

Discussion

I report here the sequence of a new crustacean FaRP: GYNRSFLRFamide. GYNRSFLRFamide is more similar in structure to the two *Homarus* peptides, TNRNFLRFamide and SDRNFLRFamide, than it is to other arthropodan peptides isolated from insects, although it lacks the asparagine present in all the group 3 peptides. Phyletically, *Homarus* and *Callinectes* are closely related. They are both decapods in the suborder Pleocyemata (Bowman and Abele, 1982). They diverge at the next taxanomic division, infraorder, *Homarus* belonging to the Astacidae, and *Callinectes* to the Brachyura. The Astacidae originated during the late Permian, while the Brachyura originated more recently, during the Jurassic, a difference of 100 million years (Schram, 1982). The class Crustacea originated in the Cambrian (Barnes, 1974), whereas the class Insecta arose much later, during the Mississippian (Meglitsch, 1967), a difference of 265 million years.

The *Drosophila* genes encode for group 2 and 3 peptides which contain asparagine and aspartic acid, respectively, in the position just adjacent to the tetrapeptide core. The codons for both of these amino acids differ by only a single nucleotide. In the blue crab, the amino acids in the same position are serine and asparagine (seen in the partial sequences), both of which

also differ by only one nucleotide in the genetic code. Therefore, the gene encoding the FaRPs in the decapod Crustacea may be similar to those of the fruit fly and the pulmonate molluscs, containing copies of multiple peptides. But the crustacean FaRPs have peptides with a C-terminal SFLRFamide, as well as NFLRFamide.

The group 1 peptides are unlike those in crustaceans and a single change in a codon cannot change a valine to either a serine or an asparagine. The group 1 and 3 peptides also have very different biological effects. In a single experiment (not shown in Table 3-3), the insect peptide pQDVDHVFLRFamide had a threshold of 10,000 times higher than that of the lobster peptide TNRNFLRFamide in stimulating the heart. This difference is reciprocal; the locust heart is potently inhibited by its native peptide, whereas TNRNFLRFamide is cardioexcitatory (Cuthbert and Evans, 1989; Robb et al., 1989).

The peptide isolation and identification data clearly indicates that several different FaRPs are present in *Callinectes sapidus*, including the new sequence GYNRSFLRFamide and several partial ones with the N-terminal sequence NFLRFamide. As with the peptides in *Homarus americanus* (Trimmer et al., 1987), they are N-terminal extensions of FLRFamide. However, there is evidence for one peptide in which a tyrosine has been substituted for the N-terminal phenylalanine

(X-X-His-Lys-Asn-Tyr-Leu-Arg-Phe) (Table 3-2). Two peptides containing the C-terminal sequence YLRFamide have been isolated from the gastropod mollusc, *Helix aspersa* (Price et al., 1990).

Many of the blue crab FaRP sequences appear to be fragmented, and Trimmer et al. (1987) also reported finding a fragment of the TNRNFLRFamide sequence. Yet different methods of extraction were employed with *Homarus* (Trimmer et al., 1987) and *Callinectes* (present study). If the crustaceans contained a hardy protease, which was not inhibited by either acetone or methanol, then some digestion of the peptides could occur during one of the extraction steps. Also, an enzyme might coelute with the peptide during the first HPLC separation and act at this point, though it would have to be acetonitrile resistant and function in the presence of TFA. An enzyme released during dissection might degrade the peptide before the pericardial organs and thoracic ganglia could be immersed in the acetone. Another possible explanation is that the Asn-Phe and Ser-Phe bonds are labile under the conditions of extraction, purification, and storage. But the nematode peptides, SDPNFLRFamide and SADPNFLRFamide were processed and purified in the same manner in this laboratory, and no fragments were observed.

The crustacean heart beat is neurogenic; a cardiac ganglion innervates the myocardium and initiates the

contractions (Maynard, 1960; Hartline, 1967; Kuramoto and Yamagishi, 1990). Nine neurons make up the lobster cardiac ganglion, five large motor neurons at its anterior end, and four small driver neurons posteriorly (Maynard, 1953; 1955; Hagiwara, 1961). Only the large neurons innervate the myocardium; the small cells send processes only to the large ones. Electrical recordings show that the small cells trigger a burst of spike activity in the large cells, suggesting that the small cells constitute the endogenous pacemaker (Friesen, 1975). Extrinsic excitatory and inhibitory neurons send their axons into the cardiac ganglion and the myocardium and regulate the beat (Hagiwara, 1961; Wiens, 1982). GYNRSFLRFamide primarily increases heart rate; its effects on muscle tone and amplitude seem to be indirect. Therefore, the peptide probably acts on the pacemaker cells in the ganglion.

Studies on the heart and cardiac ganglion have been performed with three other cardioactive products of the pericardial organ. Proctolin, another peptide found in pericardial organs, stimulates the intact, isolated lobster heart and the isolated cardiac ganglion (Miller and Sullivan, 1981; Sullivan and Miller, 1984). Serotonin increases the burst frequency of the small neurons, the heart rate, and to a lesser extent, the burst frequency of the large motor neurons (Kuramoto and Yamagishi, 1990).

Dopamine also increases the burst frequency of the cardiac ganglion (Miller et al., 1981; Cooke and Sullivan, 1982).

Peptide structure is related to the relative potency. The tetrapeptides are very weak agonists. When the asparagine is placed in position 5 (as numbered in GYNRSFLRFamide) (Table 3-3), the pentapeptide potency is only one order of magnitude less than GYNRSFLRFamide. The further addition of any amino acid, including arginine, in position 4 (as numbered in GYNRSFLRFamide) of this pentapeptide does not greatly change the potency. Arginine in position 4 is found in all native crustacean peptides and therefore might be necessary for receptor activation. When arginine is placed in position 4, and the asparagine in position 5 is replaced by other hydrophilic amino acids, like glutamine and serine, the peptides are slightly less potent than RNFLRFamide. However, when a hydrophobic amino acid like leucine is substituted for the asparagine, the potency of the resulting analog decreases by almost an order of magnitude compared to RNFLRFamide. When the asparagine is retained in position 5, but the arginine is replaced by either glutamate, which is negatively charged rather than positively charged, or glycine, which is small and uncharged, or pyroglutamate, which is large and polar, the analogs are equipotent with RNFLRFamide. When two or more amino acids are added to the N-terminal of the

pentapeptide, NFLRFamide, it becomes equipotent with GYNRSFLRFamide. Only one peptide TNRNFLRFamide is as much as one order of magnitude more potent than GYNRSFLRFamide. For full receptor activation, therefore, the peptide should have the FLRFamide core, an asparagine in position 5, and amino acids in positions 3 and 4 (as numbered in GYNRSFLRFamide).

Some of the partially sequenced crab peptides have the asparagine proximal to the FLRFamide core and are expected to have full biological activity on the heart bioassay. But GYNRSFLRFamide lacks the asparagine in position 5, and yet it is equipotent with the analogs that do possess an asparagine. In the native crab peptide, serine occupies position 5. RSFLRFamide is about half as potent as RNFLRFamide. A comparison of serine and asparagine shows that the hydrogen of the hydroxyl group in serine will have a slight positive charge, as will the nitrogen in the amide group of asparagine (Roberts and Caserio, 1977). If the amide nitrogen on the asparagine binds to a nucleophilic region on the receptor, then the hydrogen of the serine hydroxyl group might also be positioned close enough to bind in the same way. This could also explain why RQFLRFamide is also half as potent as RNFLRFamide. When glutamine is placed in position 5, its amide group is located one carbon atom farther away from the peptide backbone than in asparagine, and its

nitrogen would therefore be too far away to bind optimally to that nucleophilic region of the receptor. Thus, peptides like GYNRSFLRFamide, with serine in position 5, would be equipotent with peptides containing asparagine in position 5.

CHAPTER 4

SUMMARY AND CONCLUSIONS

Both the polychaete annelid *Nereis virens*, and the crustacean *Callinectes sapidus*, contain FMRFamide-related peptides. In *Nereis*, the native FaRP is the tetrapeptide FMRFamide, the first molluscan FaRP to be found in an extra-molluscan species. Immunohistochemically it is localized in cells and processes of the circumesophageal nerve ring and the ganglia of the ventral nerve cord, as well as the gut and other non-nervous tissues. FMRFamide relaxes the spontaneously active and electrically stimulated esophagus in a dose-dependent manner, suggesting that it is involved in regulating gastric motility. In *Callinectes*, the native FaRP is GYNRSFLRFamide. It is found in the pericardial organs and is a potent cardioexcitatory peptide. A structure-activity study revealed the requirements for full biological potency.

The polychaete peptide, FMRFamide, is identical to the tetrapeptide sequence found in all molluscs. All other extra-molluscan peptide sequences have been N-terminal extensions of the tetrapeptide core, FXRFamide (where X is L, M, or I). Annelids and molluscs have many

characteristics which suggest a common ancestry (Stasek, 1972; Field et al., 1988). The peptide sequence is consistent with this, and suggests that the tetrapeptide gene may have arisen in the primordial ancestor.

FMRFamide is found throughout the central nervous system of the worm. It is also localized in the periphery, including the body wall and digestive system. Furthermore, exogenous FMRFamide causes a dose-dependent relaxation of the isolated esophagus. These data indicate that FMRFamide is a neurotransmitter in the central and peripheral nervous system and is involved in the control of gastric motility.

Unlike the peptide in *Nereis*, the blue crab peptide, GYNRSFLRFamide is an N-terminally extended FaRP. It and the other partially sequenced peptides are similar to the other two known crustacean sequences, suggesting that there are at least three distinct sub-populations of arthropodan FaRPs: two in insects, and another in both insects and crustaceans. The crustacean peptides are also similar to the peptides found in the nematode, *Panagrellus redivivus*, SADPNFLRFamide and SDPNFLRFamide. Both of these peptides contain an asparagine in the position adjacent to the FLRFamide core; the same residue is also found in the same position in many of the crustacean and *Drosophila* peptides and affects the potency of the peptides in the crustacean heart bioassay. Nematodes are

ancient and probably evolved from the same primordial group of flatworms that gave rise to the annelids, arthropods, and molluscs (Field et al., 1988). The similarity in peptide sequence suggests that the asparagine-containing FaRPs may have developed early in evolution.

The *Callinectes* peptide, GYNRSFLRFamide, is found in the pericardial organs, part of the crustacean neuroendocrine system. It is readily accessible to the heart and causes a dose-dependent increase in heart rate. This suggests that GYNRSFLRFamide and the other native FaRPs have a role in controlling the circulation and possibly other organs of *Callinectes sapidus*.

The structure and activity study of the crustacean cardiac receptor shows that the receptor requires more than the FLRFamide tetrapeptide core. For full potency the peptide should have the sequence XXZFLRFamide, where X is any amino acid and Z is either asparagine or serine. This is unlike any other FaRP receptor studied to date, although the similarity of the crustacean peptide sequences to some *Drosophila* and nematode peptides, i.e. the C-terminal sequence NFLRFamide, may indicate a similarity in receptors.

The results of this dissertation suggests several lines of research that could be pursued. The FaRP genes of both *Nereis virens* and *Callinectes sapidus* should be

isolated and sequenced. The nereid gene may reveal other less abundant peptide sequences undetected by the peptide isolation and sequencing techniques used here. The crab gene would identify the missing amino acids in the partial sequences isolated in this study.

In *Nereis*, a structure-activity study of the esophageal relaxation response would reveal whether the annelid receptor is similar to the molluscan tetrapeptide receptor.

In *Callinectes*, a receptor binding assay should be developed to determine whether the *in vitro* bioassay data corresponds to binding affinities of the FaRPs studied.

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BIOGRAPHICAL SKETCH

Kevin Gerard Krajniak was born on June 22, 1958, in Glendale, California. There he remained until the age of eighteen when he moved south for an undergraduate education at the University of San Diego. Then, for reasons still unknown even to himself, he moved to the Great White North (Calgary, Alberta, Canada) where he saw snow for the first time in his life (up close and personal). He also completed a master's degree in animal physiology at the University of Calgary under the guidance of Dr. George Bourne by studying abalone (marine snails) in the middle of the Rocky Mountains. Naturally occurring Rocky Mountain abalones (unlike oysters) were very scarce, and this research project therefore required many extra years of effort. Having seen enough snow and temperatures well below zero, he again packed up and this time headed to the great humid south, i.e., Florida, where he worked with Dr. Michael J. Greenberg at the Whitney Laboratory. During the course of his doctoral research at the University of Florida, he studied neuropeptides in all manner of invertebrates, both crunchies and squishies, including the great sliming slug and its coiling penis,

the malevolent blue crab and its beating heart, and the ever disgusting blood- and sand- worms and their contracting entrails. Having completed his doctoral dissertation at UF, he now returns to his point of origin to study developmental neurobiology at UCLA with Dr. Jim Waschek and contemplates on what a long strange trip it has been.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michael J. Greenberg, Chairman
Professor of Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William R. Kem
Professor of Pharmacology and Therapeutics

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Thomas C. Rowe
Associate Professor of Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William E. Carr
Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ben M. Dunn
Professor of Biochemistry

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1990

Dean, College of Medicine

Dean, Graduate School